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WE, Paul B.Fisher, Dong-Chul Kang and Rahul V. Gopalkrishnan, citizens of the United States, Korea, and India, respectively, having post office addresses at 15 Gordon Place, Scarsdale, New York 10583; 200 Union Avenue, Apartment A, Rutherford, New Jersey 07070; and 302 West 79th Street, Apt. 2A, New York, New York 10024, respectively, have made an invention entitled:

USE OF MDA-5 AS AN ANTIVIRAL AND ANTIPROLIFERATIVE AGENT

of which the following is a

SPECIFICATION

This application is a continuation-in-part of International Patent Application No. PCT/US01/06960, filed February 28, 2001 and published in English on September 7, 2001 as Publication No. WO 01/64707, which is a continuation-in-part of United States Patent Application No. 09/515,363, filed February 29, 2000.

The subject matter described herein was developed at least in part using funds provided by National Institutes of Health Grants CA35675 and CA74468, so that the United States Government has certain rights to this patent application.

1. INTRODUCTION

[0001] The present invention relates to melanoma differentiation associated gene-5 (*mda*-5) nucleic acids, MDA-5 proteins, and the *mda*-5 gene promoter and related molecules, and the use of these elements to protect against or limit viral infection, to control cell proliferation and to induce apoptotic cell death. It is based, at least in part, on the discovery that MDA-5 protein exhibits ATPase activity.

2. BACKGROUND OF THE INVENTION

2.1 DIFFERENTIATION THERAPY

[0002] Terminal differentiation is essential for normal development and homeostasis (Leszczyniecka *et al.*, 2001, *Pharmacol Ther* 90(2-3):105-56). However, this process can go awry in cancer cells, resulting in uncontrolled proliferation and an inability to respond to normal growth-inhibitory signals (Fisher *et al.*,1985,

Pharmacol Ther 27(2):143-66; Waxman S., 1995, Differentiation Therapy, Waxman, ed., Sereno Symposium Publications: Rome, Italy. pp. 1-531). Moreover, as cancer cells evolve, ultimately developing new phenotypes or acquiring a further elaboration of pre-existing transformation-related properties, the expression of differentiation-associated traits often undergoes a further decline.

[0003] Based on these observations, a new rationale for cancer therapy is being explored, in which tumor cells are treated with agents that induce differentiation and a loss of cancerous properties, a strategy called "differentiation therapy" (Waxman *et al.*, 1988, In: The Status of Differentiation Therapy of Cancer, Waxman *et al.*, eds., Raven Press: New York, NY. pp. 1-422; Waxman *et al.*, 1991, In: The Status of Differentiation Therapy, Vol. 1, Waxman *et al.*, eds., Raven Press: New York, NY; Jiang *et al.*, 1994, *Mol Cell Different* 2:221-239; Waxman, 1995, Differentiation Therapy, Waxman, ed., Sereno Symposium Publications: Rome, Italy. pp. 1-531). In principle, differentiation therapy may prove less toxic than currently employed chemotherapeutic approaches, including radiation and treatment with toxic chemicals. The clinical application of differentiation therapy will require the identification of the genes and pathways involved in determining cell fate, as well as the development of appropriate *in vitro* and *in vivo* model systems for identifying and characterizing the appropriate agent or agents that can modulate differentiation in cancer cells without causing undue toxicity to normal cells.

[0004] Treatment of human melanoma cells with a combination of recombinant human fibroblast interferon (IFN-ß) and the anti-leukemic compound mezerein (MEZ), an activator of protein kinase C, results in a rapid and irreversible suppression of growth, an extinction of tumorigenic potential in nude mice, and the induction of

terminal cell differentiation (Fisher *et al.*, 1985, *J Interferon Res* 5:11-22). This process correlates with significant changes in cellular physiology, including irreversible alterations in growth, enhanced melanogenesis, morphological changes, cell surface antigen modifications, and profound variations in gene expression (Jiang *et al.*, 1994, *Mol Cell Different* 2:221-239; Leszczyniecka *et al.*, 2001, *Pharmacol Ther* 90(2-3):105-156).

[0005] When administered alone, IFN-ß or MEZ can induce specific differentiation-associated changes in melanoma cells including growth suppression, melanogenesis, and alterations in morphology. However, these changes in phenotype are fully reversible after removal of the inducer (Fisher *et al.*, 1985, *J Interferon Res* 5:11-22; Jiang *et al.*, 1993, *Mol Cell Different* 1:41-66; Jiang *et al.*, 1994, *Mol Cell Different* 2:221-239; Leszczyniecka *et al.*, 2001, *Pharmacol Ther* 90(2-3):105-56). In these contexts, this differentiation system represents an ideal model to study growth control, differentiation (both reversible and terminal), and tumor suppression.

2.2. CLONING OF MELANOMA DIFFERENTIATION ASSOCIATED GENES

[0006] To identify genes potentially involved in the process of terminal differentiation in human melanoma cells, subtraction hybridization has been employed (Jiang and Fisher, 1993, *Mol Cell Different* 1:285-299). Briefly, cDNA libraries were prepared from temporal RNA samples obtained from HO-1 human melanoma cells treated with IFN-ß + MEZ and control, untreated HO-1 cells and control cDNAs were subtracted away from differentiation inducer-treated cDNAs (*Id.*). This approach resulted in an enrichment of genes displaying elevated expression as a function of treatment with the different inducers and the induction of irreversible

growth suppression and terminal cell differentiation. Screening of the subtracted differentiation inducer-treated HO-1 cDNA library identified both known and novel cDNAs displaying elevated expression in differentiation inducer treated HO-1 cells (Jiang and Fisher, 1993, *Mol Cell Different* 1:285-299; Jiang *et al.*, 1994, *Mol Cell Different* 2:221-239; Jiang *et al.*, 1995, *Oncogene* 11(12):2477-2486; Jiang *et al.*, 1996, *Proc Natl Acad Sci USA* 93:9160-9165; Lin *et al.*, 1996, *Mol Cell Different* 4:317-333; Lin *et al.*, 1998, *Gene* 207:105-110; Huang *et al.*, 1999, *Oncogene* 18:3546-3552; Huang *et al.*, 1999, *Gene* 236:125-131).

[0007] Four classes of genes, called melanoma differentiation associated (mda) genes, have been cloned using this approach (Jiang and Fisher, 1993, *Mol Cell Different* 1:285-299). These include genes displaying elevated expression as a function of treatment with: IFN-ß and IFN-ß + MEZ (Type I mda genes); MEZ and IFN-ß + MEZ (Type II mda genes); IFN-ß, MEZ and IFN-ß + MEZ (Type III mda genes); and predominantly with IFN-ß + MEZ (Jiang and Fisher, 1993, *Mol Cell Different* 1:285-299; Jiang *et al.*, 1994, *Mol Cell Different* 2:221-239). This approach has resulted in the cloning of both known and novel genes involved in important cellular processes, including cell cycle control (mda-6/p21), interferon signaling (ISG-15, ISG-54), cancer growth control (mda-7), immune interferon response (mda-9), transcription control (*c-jun*, *jun-B*), immune recognition (HLA Class I) and cell membrane processes (α5 integrin, ßa integrin, fibronectin) (Jiang and Fisher, 1993, *Mol Cell Different* 1:285-299; Jiang *et al.*, 1994, *Mol Cell Different* 2:221-239; Jiang *et al.*, 1995, *Oncogene* 11:2477-2486; Jiang *et al.*, 1995, *Oncogene* 10:1855-1864; Jiang *et al.*, 1996, *Proc Natl Acad Sci USA* 93:9160-9165; Lin *et al.*, 1996, *Mol Cell Different* 4:317-333; Lin *et al.*, 1998, *Gene* 207:105-110).

[0008] Phage-based cDNA-subtracted libraries and a rapid subtraction hybridization (RaSH) approach have been used to define the relevant gene changes associated with induction of terminal differentiation (Jiang *et al.*, 1993, *Mol Cell Different* 1:41-66; Leszczyniecka *et al.*, 2001, *Pharmacol Ther* 90(2-3):105-156). Subtraction hybridization between a temporally-spaced differentiation inducer (IFN-ß + MEZ)-treated HO-1 human melanoma cDNA library and a temporally-spaced, untreated control HO-1 cDNA library identified a differentially-expressed 0.3 kb EST, designated melanoma differentiation associated gene-5 (*mda-5*; Jiang and Fisher, 1993, *Mol Cell Different* 1:285-299; United States Patent No. 5,643,761 by Fisher *et al.*, filed October 23, 1993 and issued July 1, 1999; International Patent Application No. PCT/US94/12160, publication No. WO 95/11986 by The Trustees of Columbia University in the City of New York, Fisher *et al.*, inventors, filed October 24, 1994). Northern blotting analysis indicated that the *mda-5* EST hybridized with an mRNA species of ~3.8 kb in IFN-ß + MEZ-treated HO-1 cells (Jiang and Fisher, 1993, *Mol Cell Different* 1:285-299; Jiang *et al.*, 1994, *Mol Cell Different* 2:221-239).

[0009] A full-length *mda*-5 cDNA (FIGURE 1; SEQ ID NO:1) has now been cloned by using a modified rapid amplification of cDNA ends (RACE) approach (International Patent Application No. PCT/US01/06960, Publication No. WO 01/64707 by The Trustees of Columbia University in the City of New York, Fisher *et al.*, inventors, filed February 28, 2001). The cDNA is 3,365 bp in length, excluding the polyA tail. Two ATTTA motifs, which are commonly found in RNA species that are rapidly turned over, are present at positions 3,225 and 3,284. A poly A signal (AATAAA) is located 23 bp upstream of the poly A tail.

[0010] In addition, a variant of *mda-5*, named *mda-5p* (SEQ ID NO:4), which contains an additional 202 bp attached to the 3' end of *mda-5*, was identified by screening a placental cDNA library (International Patent Application No. PCT/US01/06960, Publication No. WO 01/64707 by The Trustees of Columbia University in the City of New York, Fisher *et al.*, inventors, filed February 28, 2001). Since the poly A signal for *mda-5p* is also located 23 bp upstream of its poly A tail, while the open reading frame (ORF; see below) remains constant, *mda-5p* is possibly an alternatively poly-adenylated variant of *mda-5*.

[0011] An ORF is present in the *mda-5* cDNA which extends from nt 169 to 3,246, and encodes a predicted protein of 1,025 amino acids having a molecular mass of 116.7 kDa (International Patent Application No. PCT/US01/06960, Publication No. WO 01/64707 by The Trustees of Columbia University in the City of New York, Fisher *et al.*, inventors, filed February 28, 2001). The primary sequence of this predicted protein is shown in FIGURE 2 (SEQ ID NO:2). *In vitro* translation of the *mda*-5 cDNA results in a protein with an apparent molecular mass of 120 kDa and additional smaller bands, most likely truncated translation products of MDA-5.

[0012] The *mda-5* gene is an early IFN- and tumor necrosis factor-α (TNF-α)-responsive gene, the transcription of which is increased by treatment with IFN-ß and IFN-ß + MEZ. Thus, this gene may play a significant role in IFN-induced growth suppression and/or apoptosis. Identification, cloning and analysis of upstream genomic sequences has identified an *mda*-5 promoter element (FIGURE 3; SEQ ID NO:3) and has confirmed that the *mda-5* gene is responsive at the transcriptional level to induction primarily by IFN-ß and dsRNA (International Patent Application No.

PCT/US01/06960, Publication No. WO 01/64707 by The Trustees of Columbia University in the City of New York, Fisher *et al.*, inventors, filed February 28, 2001).

[0013] Profile scans of the MDA-5 protein reveal putative CARD (caspase recruitment domain) and RNA helicase motifs (International Patent Application No. PCT/US01/06960, Publication No. WO 01/64707 by The Trustees of Columbia University in the City of New York, Fisher *et al.*, inventors, filed February 28, 2001). Multiple sequence alignments of the CARD motif in MDA-5 using the ClustalW system indicate that this region most closely resembles the CARD of RAIDD, which is a component of YNF-R1-mediated apoptotic signalling pathway and which contains both a death domain and a CARD motif. RAIDD interacts with RIP through its death domain and with ICH-1 (caspase-2) probably via its CARD motif.

2.3 ANTIVIRAL CELLULAR DEFENSES STIMULATED BY IFN AND dsRNA

[0014] The cellular responses induced by IFN constitute an organism's primary defense against virus infection. Upon infection, IFN gene expression is induced, IFN-encoding RNA transcripts are translated, and IFN proteins are rapidly secreted from the cell. These IFNs may then exert a variety of effects on surrounding cells, the goal of which is to protect those cells from secondary virus infection.

[0015] A number of different IFN molecules may be produced, depending on the type of cell infected. For example, the type I IFNs, IFN-α and IFN-ß, are released from infected leukocytes and fibroblasts, respectively. Type I IFNs interact with specific receptors on the cell surface, activating a signaling cascade pathway that leads to the transcriptional induction of at least 30 genes (Sen *et al.*, 1993, *Adv Virus Res* 42:57-102). Two of these IFN-induced genes encode the enzymes 2'-5'-

oligoadenylate synthase (OAS) and protein kinase R (PKR). Both of these enzymes are activated by the binding of dsRNA molecules, including those present in the cell cytoplasm following the infection of the cell by certain RNA or DNA viruses (see below). The activation of OAS by dsRNA increases the synthesis of 2'-5' oligoadenylates, which in turn activate RNase L. Activated RNase L nonspecifically degrades single-stranded RNAs and thus limits the replication of viruses with single-stranded RNA genomes or for which single-stranded RNA molecules are formed as intermediates during replication of the virus genome (Player and Torrence, 1998, *Pharmacol Ther* 78(2):55-113).

[0016] The activation of PKR by dsRNA leads to a number of different cellular responses. For example, activation of PKR leads to induction of type I IFN gene expression (Der and Lau, 1995, *Proc Natl Acad Sci USA* 92(19):8841-8845), creating a positive-feedback loop enhancing the antiviral activities of these molecules. PKR activation also triggers the phosphorylation of several cellular proteins, including the eukaryotic translation initiation factor eIF-2-α and the inhibitor of nuclear factor-κB (IκB). Phosphorylation of eIF-2-α leads to the stabilization of the complex formed between eIF-2-α and eIF-2B, preventing further translation initiating events. Thus, activation of PKR by IFN in the presence of dsRNA blocks the further synthesis of both cellular and viral proteins (Pain, 1996, *Eur J Biochem* 236(3):747-71). Phosphorylation of IκB results in the activation of NF-κB (Kumar *et al.*, 1994, *Proc Natl Acad Sci USA* 91(14):6288-6292), which in turn induces the transcription of a host of genes whose protein products are important regulators of apoptosis, including p53, c-Myc, Fas, FasL, IRF-1 and caspase-1. Thus, PKR is an important molecule in

preventing replication of viral genomes, the synthesis of virus proteins and inducing programmed cell death in virally-infected cells.

2.4 RNA HELICASES

[0017] Although initially recognized as potent antiviral agents, it is now apparent that IFNs also induce other profound cell-type specific effects, including growth inhibition, modulation of differentiation, induction or inhibition of programmed cell death (apoptosis), and regulation of immune system genes and their expression. As introduced above, the growth inhibitory and/or apoptotic effect of type I IFNs are mediated, at least in part, by modulation of RNA translation and degradation.

[0018] Apart from PKR and OAS, described above, RNA helicases may also play important roles in diverse processes of RNA metabolism, including degradation, translation and editing. Helicases utilize the energy provided by the hydrolysis of ATP to catalyze the unwinding of nucleic acid duplexes. All of the proteins with confirmed helicase activity examined to date contain a number of conserved sequence motifs. These motifs have been used as predictors of helicase activity, leading to the identification of more than two hundred real or putative helicase proteins (Reuven *et al.*, 1995, *J Bacteriol* 177(19):5393-5400).

[0019] The large majority of helicases share at least seven motifs encoding various functional or structural domains. Examples of functional domains include the ATPase A domain, the ATPase B domain, the nucleic acid binding domain, and the helicase domain (for a review, see Lüking *et al.*, 1998, *Crit Rev Biochem Mol Biol* 33(4):259-296). Detailed comparisons of these motifs have lead to the classification of helicases into families and superfamilies (Gorbalenya and Koonin, 1993, *Curr Opin Struct Biol*

3:419-429). Superfamily II contains the RNA helicases and can be subdivided into the DEAD, DEAH and DExH families based on their deviating ATPase B domains (Lüking *et al.*, 1998, *Crit Rev Biochem Mol Biol* 33(4):259-96).

[0020] In addition to the seven domains used to classify helicases, some putative or known helicase proteins also contain domains that suggest additional functions, such as RNase III activity. RNase III degrades double-stranded RNA, and has been implicated in gene silencing by RNA interference (Bernstein *et al.*, 2001, *Nature* 409(6818):363-366). Thus, at least some helicases, through their unwinding of dsRNA, may promote the degradation of single stranded and double stranded RNA molecules.

2.4.1. VIRAL RNA HELICASES

[0021] Double-stranded polynucleotides have been shown to be present in the replicative cycle of every virus studied to date, regardless of whether its genome is double–stranded or single-stranded, or composed of DNA or RNA (Kadaré and Haenni, 1997, *J Virol* 71(4):2583-90). Thus, it is not surprising that RNA helicases of superfamily II have been identified in the genomes of many viruses, including poxviruses such as vaccinia, alphaviruses such as Semliki Forest virus (SFV), flaviviruses such as hepatitis C virus (HCV), reoviruses such as human rotaviruses A and B, and picornaviruses such as polio virus, among others.

[0022] All viral RNA helicases studied to date exhibit NTPase activity, which is an absolute requirement for RNA unwinding. In general, the enzyme activity of virus-derived RNA helicases is further stimulated by the presence of RNA, although this feature is not universal. In viruses with dsRNA genomes, there seems to be a correlation between genome size and the presence of an RNA helicase, leading some

investigators to conclude that the relative stability of the duplex forms of longer genomes must be disrupted through helicase activity to facilitate replication and translation of the virus genome.

[0023] The genomes of approximately 80% of the positive-stranded RNA viruses studied thus far also contain at least one potential helicase. In these contexts, it would appear that the helicases may function to disrupt intramolecular base-pairing within the RNA genome or to minimize the formation of RNA duplexes during replication of the genome. Other potential functions of RNA helicases in the life-cycle of viruses include: 1) mediating the separation of duplexes or regions of secondary structure so that transcription may begin, 2) assisting in proof-reading functions during polymerase function so that fidelity is maintained during replication, and 3) disrupting secondary structures in mRNA so that ribosomal attachment can occur, thereby facilitating the initiation of translation.

2.4.2 MDA-5 HELICASE MOTIFS

[0024] Sequence analysis of the MDA-5 protein revealed a RNA helicase signature domain which spans the C-terminal half of the molecule. (International Patent Application No. PCT/US01/06960, Publication No. WO 01/64707 by The Trustees of Columbia University in the City of New York, Fisher *et al.*, inventors, filed February 28, 2001). MDA-5 contains four unique features that could mediate functional divergence from other RNA helicases (International Patent Application No. PCT/US01/06960, Publication No. WO 01/64707 by The Trustees of Columbia University in the City of New York, Fisher *et al.*, inventors, filed February 28, 2001). The CARD domain of MDA-5 in its N-terminal region is not found in any previously

identified helicase. The ATPase A motif of MDA-5 is unique and contains the region LPTGSGKT as opposed to the sequence found in other RNA helicases (GXXGXGKT). International Patent Application No. PCT/US01/06960 suggests that because of this sequence divergence, MDA-5 may not bind ATP effectively, and therefore may be an ATPase defective helicase, or may require a different energy source and/or metals for activity.

3. SUMMARY OF THE INVENTION

[0025] The present invention relates to *mda*-5 nucleic acids, MDA-5 proteins, the *mda*-5 promoter, and related molecules, and the use of each of these elements in protecting against or limiting viral infection, controlling cell proliferation, and promoting apoptosis. It is based upon the invention contained in International Patent Application No. PCT/US01/06960, Publication No. WO 01/64707 by The Trustees of Columbia University in the City of New York, Fisher *et al.*, inventors, which is incorporated by reference in its entirety herein, and further on the discovery that, contrary to earlier hypotheses based on the amino acid sequence of MDA-5, the protein is not a defective ATPase but rather exhibits ATPase activity. This supports the role of MDA-5 as a RNA helicase protein and consequently its use in promoting RNA degradation, for example in the context of an antiviral defense, limitation of cell proliferation, or induction of apoptosis.

4. BRIEF DESCRIPTION OF THE FIGURES

[0026] FIGURE 1. *mda-5* cDNA sequence (SEQ ID NO:1).

[0027] FIGURE 2. MDA-5 protein sequence (SEQ ID NO:2).

[0028] FIGURE 3. *mda*-5 gene promoter sequence (SEQ ID NO:3)

[0029] FIGURE 4. Sequence alignment of putative RNA helicases. Clustal W alignment of helicase domains of putative RNA helicases that share the RNA helicase motifs with *mda*-5. Conserved residues in DExH group RNA helicase defined in Jankowsky and Jankowsky , 2000, *Nucleic Acids Res* 28:333-334, are aligned with consensus sequence (uppercase Roman numeric). Those underlined and marked with lowercase Roman numeric are for conserved motifs in this subgroup. Asterisks (*) = identical residues; colons (:) = conserved substitutions; dots (.) = semiconserved substitutions.

[0030] FIGURE 5(A-D). Expression of *mda*-5. Northern blot analyses of *mda*-5 after treatment with melanoma differentiation-inducing reagents (A) and growth factors (B) are shown. HO-1 cells were treated with the indicated reagents for 24 hours. The concentrations in A are: control (CTL); 0.1% DMSO; 10 ng/ml MEZ; 2,000 units/ml IFN-ß; 2,000 units/ml IFN-ß + 10 ng/ml MEZ; 100 units/ml IFN-γ; 100 units/ml IFN-γ + 10 ng/ml MEZ; serum-free medium (D-O); 2.5 μM *all-trans* retinoic acid: 3 μM mycophenolic acid (MPA); 16 nM 12-O-tetradecanoylphorbol-13-acetate (TPA); 1 mM 3'-5' cAMP; 1 mM 8-bromo-3'-5' cyclic adenosine monophosphate (8-Br-cAMP); and 10 ng/ml methyl methanesulfonate (MMS). The reagents in (B) are: CTL, control; 1,000 units/ml IFN-α; 1,000 units/ml IFN-μ; 1,000 units/ml IFN-γ; 1 ng/ml IL-6; 10 ng/ml epidermal growth factor (EGF); 10 ng/ml

transforming growth factor-α (TGF-α); 2.5 ng/ml transforming growth factor-ß (TGF-ß); 1 ng/ml TNF-α; and 10 ng/ml platelet-derived growth factor (PDGF). (C) Nuclear run-on assays for *mda*-5. Nuclei were prepared from HO-1 melanoma cells treated with the indicated reagents for 4 hours. Blots were prepared and hybridized as described in Materials and Methods. gapdh = glyceraldehyde-3-phosphate dehydrogenase.(D) PKC inhibitors in *mda*-5 expression. RNA samples were extracted from HO-1 melanoma cells pretreated with 50 nM staurosporine or 0.2 μM Ro31-8220 (Calbiochem) for 30 min and treated with the indicated reagents for 8 h. RNA sample preparation and Northern hybridization were performed as described in Materials and Methods.

[0031] FIGURE 6(A-B). Protein expression of *mda*-5 and intracellular localization. (A) Protein expression from *mda*-5 cDNA after transient transfection. Protein extracts were prepared from 293 cells transiently transfected with the indicated expression vector and resolved in 9% SDS/PAGE. Western blot analysis was performed with specified antibodies. ctl = control. (B) Intracellular localization of *mda*-5 protein. Transiently transfected 293 cells on cover slips with the indicated fusion protein constructs were mounted and photographed using fluorescent confocal microscopy (x400).

[0032] FIGURE 7(A-B). Effect of ectopic expression of *mda*-5 on HO-1 cells. Cells were transfected with the indicated expression vector, replated 2 days later, and selected with G418. (A) Representative colony-forming assays. G418-resistant colonies transfected with the indicated expression vectors are shown. Ctl, control; AS, antisense. (B) Quantitation of the effect of ectopic expression of *mda*-5 on colony formation. Giemsa-stained G418-resistant colonies containing more than ≈50 cells

were counted. The results are the mean ± standard error from three independent transfections (three plates for each transfection) with two different plasmid batches.

[0033] FIGURE 8(A-D). ATPase activity of MDA-5. (A) Electrophoretogram of purified proteins. GST and GST-MDA-5 were resolved on 9% SDS/PAGE and stained with Coomassie brilliant blue. (B) Effect of divalent metal ions and RNA concentration on MDA-5 ATPase activity. ATPase activity assay was performed with variable poly(I)•poly(C) concentrations (0.038, 0.375, 3.75, 37.5, and 375 ng/μl). $MnCl_2$ (3 mM,■) substituted for $MgCl_2$ (3 mM, ◆). The results were quantitated by PhosphorImager. The data shown are the mean ± SD from two experiments. Specific activity is nmol•min•μg of protein. (C) Lineweaver-Burk plot of the effect of [ATP] on MDA-5 ATPase activity. ATPase activity was measured with various ATP concentrations (15.6, 31.3, 62.5, 125, 250, and 500 μM) for 20 min in a 37°C air incubator and quantitated by PhosphorImager. The result shown is the mean ± SD from three independent experiments. (D) Autoradiogram of MDA-5 ATPase assay with various RNA species. The indicated types of RNA (20 ng/μl) were added in standard reaction mixture, and the results presented were obtained by exposing a TLC plate to Kodak X-Omat film. D.W., distilled water.

[0034] FIGURE 9. Nucleic acid sequence of *mda-5p* (SEQ ID NO:4).

[0035] FIGURE 10. Amino acid sequence of protein encoded by *mda-5p* (SEQ ID NO:7).

[0036] FIGURE 11. Nucleic acid sequence encoding an MDA-5 protein (SEQ ID NO:8).

[0037] FIGURE 12. Amino acid sequence (SEQ ID NO:9) of MDA-5 protein encoded by SEQ ID NO:8.

5. DETAILED DESCRIPTION OF THE INVENTION

[0038] For purposes of clarity, and not by way of limitation, the detailed description is divided into the following subsections:

MDA-5 related molecules;

measuring ATPase activity;

(iii) use of MDA-5 activity as an antiviral agent; and

(iv) use of MDA-5 activity as an antiproliferative agent.

5.1 MDA-5 RELATED MOLECULES

[0039] A "*mda*-5 nucleic acid" according to the invention is a nucleic acid which comprises (i) the nucleic acid sequence as set forth in FIGURE 1 (SEQ ID NO:1), or (ii) a nucleic acid which encodes a protein having an amino acid sequence as set forth in FIGURE 2 (SEQ ID NO:2); or (iii) a nucleic acid which hybridizes to a nucleic acid molecule having the sequence set forth in FIGURE 1 (SEQ ID NO:1) under stringent conditions; and/or (iv) a nucleic acid which is at least 90 percent homologous to a nucleic acid molecule having a sequence as set forth in FIGURE 1 (SEQ ID NO:1), as determined by a standard homology-assessing software program such as BLAST.

[0040] For example, the following would be an example of stringent hybridization conditions (which may include wash conditions). Maximum hybridization specificity for DNA samples immobilized on nitrocellulose filters may be achieved through the use of repeated washings under conditions of high stringency, such as when the

washings are done in a solution comprising 0.1- 0.2 X SSC (15-30 mM NaCl, 1.5-3 mM sodium citrate, pH 7.0) and 0.1% SDS (sodium dodecylsulfate) at temperatures of 65-68°C or greater (Current Protocols in Molecular Biology, Volume I. Ausubel *et al.*, eds. John Wiley:New York NY, pp. 2.10.1-2.10.16. 1989 with annual updating). For DNA samples immobilized on nylon filters, a stringent hybridization washing solution may comprise 40 mM $NaPO_4$, pH 7.2, 1-2% SDS and 1 mM EDTA. Again, a washing temperature of at least 65-68°C is recommended, but the optimal temperature required for a truly stringent wash will depend on the length of the nucleic acid probe, its GC content, the concentration of monovalent cations and the percentage of formamide, if any, that is contained in the hybridization solution (Current Protocols in Molecular Biology, Volume I. Ausubel *et al.*, eds. John Wiley:New York NY, pp. 2.10.1-2.10.16. 1989 with annual updating).

[0041] The term "*mda*-5 nucleic acid" can refer to a cDNA sequence (for example, SEQ ID NO:1), to a genomic DNA sequence, or to an RNA molecule (including an antisense RNA molecule). A *mda*-5 nucleic acid may be comprised in a vector molecule, for example a virus (which may be a defective or non-defective virus) or a plasmid, phage, cosmid, etc.. Additional elements to aid in replication and/or expression may be further comprised in the vector. A *mda*-5 nucleic acid may, for example, be operably linked to a promoter element which may be a *mda*-5 promoter or a heterologous promoter. A *mda*-5 nucleic acid may be of human or non-human species origin.

[0042] The term "*mda*-5 nucleic acid" further encompasses a variant of the *mda-5* nucleic acid having SEQ ID NO:1; this variant is named *mda-5p* and contains an additional 202 bp attached to the 3' end of *mda*-5. The sequence of *mda-5p* is set

forth in FIGURE 9 (SEQ ID NO:4). Yet another variant sequence for an *mda*-5 nucleic acid is set forth in FIGURE 11 (SEQ ID NO:8).

[0043] A MDA-5 protein according to the invention is a protein which comprises (i) a protein having the amino acid sequence set forth in FIGURE 2 (SEQ ID NO:2); (ii) a protein having the amino acid sequence set forth in FIGURE 10 (SEQ ID NO:7): (iii) a protein having the amino acid sequence set forth in FIGURE 12 (SEQ ID NO:9) or (ii) a protein encoded by a nucleic acid which hybridizes to a nucleic acid molecule having a sequence as set forth in FIGURE 1 (SEQ ID NO:1) under stringent conditions; and/or (iii) a protein which is at least 90 percent homologous to a protein having an amino acid sequence as set forth in FIGURE 2 (SEQ ID NO:2) as determined by a standard homology-assessing software program such as BLAST. A MDA-5 protein may be of human or non-human species origin.

[0044] The present invention further provides for antibody molecules which specifically bind to a MDA-5 protein, which may be monoclonal antibodies, polyclonal antibodies, single chain antibodies, or fragments thereof, generated according to methods known in the art.

[0045] A "*mda*-5 promoter" is a nucleic acid which comprises (i) a nucleic acid comprising the sequence set forth in FIGURE 3 (SEQ ID NO:3); or (ii) a nucleic acid which hybridizes to a nucleic acid having the sequence set forth in FIGURE 3 (SEQ ID NO:3) under stringent conditions; and/or (iii) a nucleic acid which is at least 90 percent homologous to a nucleic acid having a sequence as set forth in FIGURE 3 (SEQ ID NO:3), as determined by a standard homology-assessing software program such as BLAST. A *mda*-5 promoter of the invention may be comprised in a vector

molecule, such as a virus (defective or non-defective) or plasmid, or phage, or cosmid vector, and/or may be operably linked to a gene of interest. Suitable genes of interest include, but are not limited to, a *mda*-5 nucleic acid, or a protein which binds to a MDA-5 protein, or a tumor suppressor gene such as (but not limited to) p21, the retinoblastoma tumor suppressor gene, or p53, or a reporter gene, for example, but not limited to, luciferase or beta glucuronidase.

5.2 MEASURING ATPase ACTIVITY

[0046] ATPase activity may be measured using any method known in the art. In a preferred specific non-limiting embodiment of the invention, ATPase may be measured using the method set forth in the working example below, as follows.

[0047] An ATPase assay may be performed in a 10-μl reaction mixture containing 50 mM Mops, pH 6.5, 3 mM $MgCl_2$, 2 mM DTT, 0.5 mM ATP, 5 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP (6,000 Ci/mmol; Amersham Pharmacia), DNA (sonicated salmon sperm) or RNA, and test protein (0.2 μg) in a 37°C air incubator. Poly(I)•poly(C) (Amersham Pharmacia) or other RNA which has been reextracted with acid phenol /chloroform and precipitated may be used as a substrate for the reaction; for example, variable poly(I)•poly(C) concentrations (0.038, 0.375, 3.75, 37.5, and 375 ng/μl) may be used. The results may be quantitated by PhosphorImager. The reaction may be initiated by addition of the ATP mixture and the dissociated P_i may be resolved in polyethylene imine cellulose TLC plates as described (Wagner *et al.*, 1998; *EMBO J* 17:2926-2937).

5.3 USE OF MDA-5 ACTIVITY AS AN ANTIVIRAL AGENT

[0048] The present invention provides for methods of protecting against or limiting viral infection in a subject comprising administering, to the subject, an agent which increases the level of MDA-5 protein activity in the subject. "Protecting against contracting a viral infection" does not require that there be absolute protection; for example, substantially decreasing the likelihood of infection, for example reducing the risk of infection, with exposure, at least 2-fold, is considered protection. "Limiting viral infection", as defined herein, refers to any one or more of decreasing the number of cells infected by a virus within a subject or among several subjects; decreasing the cytopathic effect caused by a virus, decreasing the amount of viral replication, decreasing the clinical severity of a viral infection, and/or decreasing the period of time that a subject suffers from a viral infection. "Increasing the level of MDA-5 protein activity in the subject" encompasses increasing the level of MDA-5 protein activity in at least some but not necessarily all cells, tissues, and/or fluids of the subject. The level of MDA-5 protein activity may be measured directly or indirectly, for example, but not by way of limitation, by measuring the amount of MDA-5 protein, the amount of ATPase activity, and/or the amount of MDA-5 encoding mRNA.

[0049] In a more specific, non-limiting embodiment, the present invention provides for a method of protecting against or limiting viral infection in a subject comprising administering, to the subject, a therapeutically effective amount of an agent which increases MDA-5 protein-mediated RNA degradation in the subject.

[0050] In another non-limiting embodiment, the present invention provides for methods of protecting against or limiting viral infection in a subject comprising

administering, to the subject, a therapeutically effective amount of an agent which increases ATPase activity in the subject.

[0051] Examples of agents which may be used to increase the level of MDA-5 protein, or MDA-5 protein-mediated RNA degradation, or ATPase activity in a subject include, but are not limited to, (i) MDA-5 proteins; (ii) *mda*-5 nucleic acids, in expressible form, which encode MDA-5 proteins; (iii) agents which increase the activity of an *mda*-5 promoter or that stabilize MDA-5 encoding mRNA; (iv) agents which increase the RNA degradation activity of MDA-5 protein; and (v) agents which increase ATPase activity of MDA-5 protein.

[0052] As non-limiting examples within each of these categories, consider the following:

[0053] A therapeutic amount of MDA-5 protein may be administered to a subject by any suitable route, including intravenously, intramuscularly, orally, or by inhalation, so as to supply cells which are infected or at risk of being infected with the protein. The MDA-5 protein may be comprised in a vehicle which aids cellular uptake and/or inhibits protein degradation, for example, in liposomes or microspheres. As a specific non-limiting embodiment, an effective amount of MDA-5 protein may be administered to a subject who has been or may be in contact with a virus spread by the respiratory route in the form of an inhaled aerosol spray.

[0054] A *mda*-5 nucleic acid may be administered in expressible form, for example, in the form of "naked DNA" or comprised in a vector, for example a viral vector. In particular non-limiting embodiments the virus may be an adenovirus, such as, for example, a replication-defective adenovirus. The *mda*-5 nucleic acid may be operably

linked to a suitable promoter, which may be an *mda*-5 promoter or a heterlogous promoter, such as the cytomegalovirus immediate early promoter, the Rous sarcoma virus long terminal repeat promoter, the human elongation factor 1α promoter, the human ubiquitin c promoter, etc.. It may be desirable, in certain embodiments of the invention, to use an inducible promoter. Non-limiting examples of inducible promoters include the murine mammary tumor virus promoter (inducible with dexamethasone); commercially available tetracycline-responsive or ecdysone-inducible promoters, etc.. As a specific non-limiting embodiment, an effective amount of an *mda*-5 nucleic acid, operably linked to a promoter sequence, comprised in a viral vector such as a replication defective adenovirus vector, may be administered to a subject who has been or may be in contact with a virus spread by the respiratory route in the form of an inhaled aerosol spray. For example, the amount of virus administered may be 10^9 - 10^{13} plaque forming units.

[0055] In further embodiments of the invention, an effective amount of a second agent may be administered to the subject before, during, or after administration of MDA-5 protein or *mda*-5 nucleic acid. The second agent is defined as a facilitator of MDA-5 activity. Examples include, but are not limited to, interferon alpha, interferon beta, interferon gamma, poly(I)(C) and tumor necrosis factor alpha. In additional embodiments, a third agent which promotes *mda*-5 activity may be additionally administered; for example, but not by way of limitation, if one of the above interferons is used as second agent, another one of these interferons may be used as third agent, or mezerein may be used.

[0056] In still further embodiments of the invention, whether or not a second and/or third agent is used, an effective amount of an antiviral agent is administered to

improve the net antiviral defense. The amount of antiviral activity achieved is greater that the amount associated with the antiviral agent used without an *mda*-5 related molecule. Examples of antiviral agents include, but are not limited to, (i) amantadine, rimantidine, and sialic acid analogues such as zanamivir or other inhibitors of influenza sialidase for the specific inhibition of influenza viruses; (ii) Aayclovir (Zovirax), ganciclovir, valacyclovir (Valtrex), lamivudine (3TC; Epivir) or other nucleoside analogs such as vidarabine for the treatment of herpesvirus infections (HSV-1, HSV-2 , VZV (chicken pox), EBV and CMV); (iii) foscarnet (Foscavir), a pyrophosphate analog, may be useful in the treatment of the above-mentioned herpesviruses, and also may useful in the treatment of retroviruses including HIV-1; (iv) zidovudine (Retrovir or AZT), didanosine, zalcitabine and related purine analogues such as ribavirin, which are inhibitors of viral reverse transcriptase, for the treatment of retroviruses including HIV-1 (ribavirin is also useful in the treatment of infections caused by arenaviruses (*e.g.* Lassa fever) or bunyaviruses (*e.g.* Hantavirus), respiratory syncytial virus (RSV) and in the treatment of Hepatitis C; the non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine (Viramune) may also be useful in the treatment of HIV-1); (v) indinavir (Crixivan), saquinavir (Fortovase) and other HIV protease inhibitors for the treatment of retroviruses including HIV-1; (vi) monoclonal antibody inhibitors of ICAM-1 for the treatment of rhinoviruses, the most common cause of colds; and (vii) zintevir, an HIV integrase inhibitor, for the treatment of HIV-1.

[0057] Many of the above-mentioned drugs can be used in combination to achieve anti-viral effects that are synergistic over those achieved by single-drug therapy, although the choice of combinations must be judicious.

[0058] In alternative embodiments of the invention, an agent which activates an *mda*-5 promoter may be used to increase transcription of the endogenous *mda*-5 gene. Among the examples of such agents are certain second agents, facilitators of MDA-5 activity, described above, such as interferon alpha, interferon beta, interferon gamma, tumor necrosis factor alpha, and poly IC. According to this set of embodiments they are administered without an *mda*-5 nucleic acid or an MDA-5 protein, although administration of an *mda*-5 promoter activating agent may be combined with one or more other facilitator of MDA-5 activity which does not act at the transcriptional level, and/or with one or more antiviral agent.

[0059] Further agents which activate an *mda*-5 promoter may be identified and used according to the invention. The invention provides for an assay for identifying an activator of an *mda*-5 promoter, comprising (i) preparing a nucleic acid construct having an *mda*-5 promoter operably linked to a reporter gene (defined herein as a gene having a detectable product) for example, but not limited to, a luciferase gene; (ii) introducing the construct into a cell, such as a eukaryotic cell, for example, but not limited to, an HO-1 cell; (iii) exposing the cell to a test agent; (iv) measuring the amount of reporter gene product produced by a cell exposed to the test agent; and (v) comparing the amount of reporter gene product produced by a cell exposed to the test agent to the amount of reporter gene product produced by a cell containing the *mda*-5 promoter carrying construct which has not been exposed to the test agent. To provide an adequate control, the cell (generally in a cell culture or organism) exposed to the test agent and the cell not exposed to the test agent should otherwise be maintained under the same or similar conditions. An increase in the level of reporter gene product

in a cell exposed to the test agent has a positive correlation with an increase in *mda*-5 promoter activity.

[0060] In still further embodiments of the invention, a therapeutic amount of an agent which increases the RNA degradation activity of MDA-5 protein may be administered to a subject. The present invention further provides for methods of identifying agents which increase the RNA degradation activity of MDA-5 protein. For example, proteins which form physical associations with MDA-5 may be identified, for example, by a yeast two-hybrid assay, or by computerized analysis which predicts interactions between proteins. After identifying a protein which associates with MDA-5, the ability of the identified protein to degrade RNA either alone or in combination with MDA-5 may be tested.

[0061] The present invention further provides for the administration of agents which increase ATPase activity, for example, agents which increase the ATPase activity of MDA-5. The present invention provides for assays for identifying such agents, which comprise combining, in an ATPase assay, MDA-5 protein and a test agent, measuring the ATPase activity of MDA-5 plus test agent, and comparing the resulting ATPase activity to the ATPase activity of the same amount of MDA-5 in the absence of the test agent.

[0062] Examples of infections which may be treated by the foregoing methods are those caused by 1) picornaviruses, a family of viruses that includes polioviruses, Coxsackie viruses, rhinoviruses, enteroviruses, and hepatitis A virus, which can cause poliomyelitus, meningitis, and hepatitis (hepatitis A) in humans, 2) caliciviruses, a family of viruses that include the mild gastroeneteritis-inducing Norwalk group of

viruses, 3) astroviruses, which can cause severe gastroenteritis, 4) togaviruses, a family of viruses that includes the alphaviruses, the cause of polyarthritis and a variety of encephalitidies including Eastern and Western equine encephalitis, and rubiviruses, the cause of rubella (German measles), 5) flaviviruses, a family of viruses including flaviviruses, pestiviruses and hepatitis C virus, which can cause St. Louis and West Nile encephalitis among others, tick borne encephalitis, Dengue fever, yellow fever, hepatitis (hepatitis C), and some types of hemorrhagic fevers, 6) coronaviruses, a family of viruses including the coronaviruses, a cause of colds in humans and feline infectious peritonitis in cats, and the toroviruses, 7) arteriviruses, 8) paramyxoviruses, a family of viruses including paramyxoviruses, rubulaviruses, morbilliviruses and pneumonoviruses, which can cause measles and mumps, 9) rhabdoviruses, a family of viruses including rhabdoviruses, vesiculoviruses, lyssaviruses, ephemeroviruses, cytorhabdoviruses and nucleorhabdoviruses, which can cause vescicular stomatitis and rabies among other diseases, 10) filoviruses, notorious as the cause of Ebola and related hemorrhagic fevers, 11) orthomyxoviruses, a family of viruses including influenza A, B and C viruses, major causative agents of influenza infections, 12) bunyaviruses, a family of viruses including bunyaviruses, phleboviruses, nairoviruses, hantaviruses and tospoviruses, viruses associated with the hemorrhagic Hanta fever, encephalitis, and Rift Valley fever, 13) arenaviridae, the cause of Lassa fever and hemorrhagic fevers such as Bolivian, Argentinian or Venezualan hemorrhagic fevers, 14) reoviruses, a family of viruses including orthoreoviruses, responsible for a host of respiratory and enteric infections, orbiviruses, responsible for Colorado tick fever, rotaviruses, a major cause of morbidity stemming from persistent diarrhea in the developing world, coltiviruses, aquareoviruses, cypoviruses, phytoreoviruses, fijiviruses and orzyaviruses 15)

birnaviruses, a family of viruses including aquabirnaviruses, avibirnaviruses and entomobirnaviruses, 16) retroviruses, a family of viruses containing lentiviruses such as HIV-1 and –2, the causative agent in AIDS, spumaviruses and a range of retroviruses including oncogenic viruses such as the leukemia-inducing HTLV family of viruses and type A, B, C or D retroviruses, 17) hepadnaviruses, a family of viruses including orthohepadnaviruses and avihepadnaviruses, the cause of hepatitis B, 18) circoviruses, 19) parvoviruses, a family of viruses including the chordoparvovirus and entomoparvovirus subfamily comprising erythroviruses, dependoviruses, entomoparvoviruses, densoviruses, iteraviruses and contraviruses, 20) papovaviruses, a family of viruses including papillomaviruses, the cause of warts and some cancers of the cervix and other gential regions, and polyomaviruses, the cause of progressive multifocal leukoencephalopathy and some human malignancies, 21) adenoviruses, a family of viruses including mastadenoviruses and aviadenoviruses, which can cause flu-like respiratory infections in humans, 22) herpesviruses, a family of viruses including the alphaherpesvirus, betaherpesvirus, and gammaherpesvirus subfamilies comprising simplexviruses, varicelloviruses, cytomegaloviruses, muromegaloviruses, roseoloviruses, lymphocryptoviruses and rhadinoviruses, the cause of chicken pox, recurring infections of the oral cavity or the genital tract, mononucleosis and some human malignancies including Burkitt's lymphoma and Hodgkin's disease, 23) poxviruses, a family of viruses including the chordopoxvirus and entemopoxvirus subfamilies comprising orthopoxviruses, the source of smallpox infection, parapoxviruses, avipoxviruses, capripoxviruses, lepropoxviruses, suipoxviruses, molluscipoxviruses, yatapoxviruses, and entomopoxviruses A, B and C, 23) unnamed viruses of the iridovirus family and those causing African Swine Fever, and 24)

unclassified human and animal viruses including Borna Disease virus, hepatitis E and X viruses and unclassified arboviruses.

[0063] In further embodiments, where a virus produces a helicase, providing increased levels of MDA-5 by one of the foregoing methods may be used to compete with the viral helicase, which would be desirable where this competition would result in inhibition of the viral helicase and have a negative effect on viral replication and/or pathogenesis. Such negative competition would occur, for example, where the viral helicase has greater ATPase activity relative to MDA-5, and/or where the viral helicase has a different binding affinity for target RNA relative to MDA-5. Such characteristics may be evaluated using standard laboratory techniques. Examples of viruses which encode helicase molecules are poxviruses such as vaccinia, alphaviruses such as Semliki Forest virus (SFV), flaviviruses such as hepatitis C virus (HCV), reoviruses such as human rotaviruses A and B, and picornaviruses such as polio virus, among others.

5.4 USE OF MDA-5 ACTIVITY AS AN ANTIPROLIFERATIVE AGENT

[0064] The present invention provides for methods of inhibiting cell proliferation and/or promoting apoptosis of a cell population or in a subject comprising administering, to the cell population or subject, an agent which increases the level of MDA-5 protein activity in the subject. Inhibiting cell proliferation and/or promoting apoptosis means decreasing the increase in the number of cells in a population over a time interval, relative to a control population in which the level of MDA-5 protein has not been increased over the same length of time, by at least about 20 percent. "Increasing the level of MDA-5 protein in the subject" encompasses increasing the

level of MDA-5 protein in at least some but not necessarily all cells, tissues, and/or fluids of the subject.

[0065] In related embodiments, the present invention provides for methods of inhibiting tumor growth in a subject comprising administering, to the subject, an effective amount of an agent which increases the level of MDA-5 protein activity in the subject.

[0066] Examples of agents which may be used to increase the level of MDA-5 protein activity include, but are not limited to, (i) MDA-5 proteins; (ii) *mda*-5 nucleic acids, in expressible form, which encode MDA-5 proteins; (iii) agents which increase the activity of an *mda*-5 promoter or that stabilize MDA-5 encoding mRNA; and (iv) agents which increase the RNA degradation activity of MDA-5 protein. These agents have been described in the foregoing section.

[0067] In particular embodiments, it may be desireable to co-administer an effective amount of an interferon, such as interferon alpha, interferon beta, or interferon gamma.

[0068] The foregoing agents may be administered in conjunction with one or more additional anti-neoplastic agent, including, but not limited to, a chemotherapeutic agent, immunotherapy, radiation therapy, and the like.

[0069] Examples of malignancy which may be treated according to the present invention include, but are not limited to, melanoma, glioblastoma multiforme, neuroblastoma, astrocytoma, osteosarcoma, breast cancer, cervical cancer, colon cancer, lung cancer, Kaposi's sarcoma, hairy cell leukemia, nasopharynx cancer, ovarian cancer, and prostate cancer.

[0070] The present invention also provides for the use of MDA-5 to sensitize a cell to the growth inhibitory and/or apoptosis-promoting effect(s) of a protein kinase C inhibitor (including, but not limited to, mezerein). According to such embodiments, one of the foregoing agents which increase MDA-5 protein activity may be administered prior to, coincident with, or after administering a protein kinase C inhibitor. Such methodology may also be used to eliminate virus-infected cells. Moreover, new inhibitors of protein kinase C may be identified by their ability to inhibit cell proliferation/promote apoptosis in the context of increased MDA-5 protein activity.

[0071] In still further embodiments, a *mda*-5 promoter may be used to deliver and express genes targeting virally infected cells for destruction. For example, the *mda*-5 promoter could be used in a virus comprising a first cancer-inhibitory gene (*e.g.*, p53, *mda*-7) operably linked to a promoter selectively active in cancer cells (*e.g.*, PEG-3), and further comprising a second cancer-inhibitory gene (*e.g.*, IL-2, interferon gamma, interleukin 12) operably linked to a *mda*-5 promoter. Such a virus would have cancer targeted specificity (due to the first promoter) and IFN or similar inducing agent induction potential (due to the *mda*-5 promoter, thereby augmenting the killing of cancer cells.

6. WORKING EXAMPLE

6.1 MATERIALS AND METHODS

[0072] Cell Cultures. HO-1 human melanoma cells and 293 cells were grown as described (Fisher *et al.*, 1985, *J Interferon Res* 5:11-22). Sf9 cells were cultured in TNM-FH medium (Mediatech Laboratories, Cody, NY) supplemented with 10% FBS and penicillin/streptomycin (100 units/100 µg/ml) at 27°C in a humidified incubator.

[0073] Cloning and Sequencing of *mda*-5. A partial *mda*-5 cDNA (3', 1.8 kb) was cloned by screening a human placental cDNA library (CLONTECH; Ausubel *et al.*, 1992. Short Protocols in Molecular Biology. Wiley:New York). The remaining 5' region of the *mda*-5 cDNA (1.5 kb) was obtained by using a modified rapid amplification of cDNA ends approach. This approach involved an anchor primer instead of poly(A) or (G) tailing designed to anneal to the 5' end of the *mda*-5 cDNA, followed by second-strand synthesis and subsequent PCR using an *mda*-5-specific reverse transcription primer to generate a full-length *mda*-5 cDNA.

[0074] Northern Blot Analyses and Nuclear Run-On Assays. RNA preparation and Northern blot hybridization were performed as described (Ausubel *et al.*, 1992. Short Protocols in Molecular Biology. Wiley:New York). Northern blots were probed with a ^{32}P-labeled 2.5-kb EcoRI fragment of *mda*-5 cDNA and a 0.7-kb glyceraldehyde-3-phosphate dehydrogenase fragment. Nuclear run-on assays were performed as described (Ausubel *et al.*, 1992. Short Protocols in Molecular Biology. Wiley:New York). Probes used for nuclear run-on assays included: *mda*-5 5', 9-837 bp; *mda*-5 3', 2,531-3,365 bp; and a glyceraldehyde-3-phosphate dehydrogenase fragment (0.7 kb). Autoradiograms were quantitated by densitometry using a Molecular Dynamics densitometer.

[0075] *mda*-5 Expression Vectors. A hemagglutinin (HA)-tagged *mda*-5 fragment was obtained by reverse transcription-PCR (GCCACCATGTACCCATACGACGTCCCAGACTACGCTATGTCGAATGGGTA TTCCACAGACG/TCACTAATCCTCATCACTAAATAAACAGC; SEQ ID NO:5) and was cloned into the EcoRV site of pcDEF3 with expression regulated by the EF-1α promoter. An antisense *mda*-5 expression vector was constructed by cloning the

EagI/SpeI *mda*-5 genomic DNA (3.8-kbp) fragment from a bacterial artificial chromosome clone into the SpeI/NotI site of pcDEF3. The genomic DNA fragment consists of the first exon and part of the first intron. A green fluorescent protein (GFP)-*mda*-5 fusion expression vector was constructed by ligation of a reverse transcription-PCR-derived *mda*-5 cDNA product (ATGTCGAATGGGTATTCCACAGACG/TTTTTTTTTTTTCAGAGTAAAACAA TC; SEQ ID NO:6) into the SrnaI site of pEGFP-C2 (CLONTECH).

[0076] Western Blot Analysis and Fluorescent Confocal Microscopy. Transient transfections were performed by using SuperFect (Qiagen, Chatsworth, CA) as described in the manufacturer's protocol. Ten micrograms of supercoiled plasmid DNA (pcDEF3, pcDEF3/HA-*mda*-5, pEGFP-C2, and pEGFP-C2/*mda*-5) were transfected into ≈70% confluent 293 cells and plated 1 day before transfection in a 10-cm plate, and cells were harvested 2 days after transfection. Protein sample preparation and Western blotting were performed as described in Jiang et al., 1995, Oncogene 11:2477-2486. MDA-5 fusion proteins were probed with either α-HA antibody (Roche Diagnostics) or α-GFP antibody (CLONTECH) and with horseradish peroxidase-conjugated anti-Mouse IgG (Sigma) and detected by ECL (Amersham Pharmacia). For intracellular localization, 10^5 cells per well were seeded in 6-well plates containing a cover glass and transfected with the indicated plasmids (pEGFP-C2 and pEGFP-C2/*mda*-5, 2.5 μg per well). For fluorescence microscopy, the cover glass containing transfected cells was washed with PBS and mounted onto a glass slide with mounting medium. The cells were observed by fluorescent confocal microscopy.

[0077] Colony-Forming Assays. HO-1 melanoma cells were plated at 8 x 10^5 in a 6-cm dish 1 day before transfection with 5 μg of supercoiled plasmid DNA. Two days after transfection, cells were trypsinized and replated at 10^5 cells per 6-cm dish with complete medium containing 750 μg of G418/ml. The G418-containing medium was replaced once a week for 3 weeks. Cells were fixed with methanol (-20°C) and stained with Giemsa (Sigma). Colonies with more than ≈50 cells were enumerated.

[0078] Purification of Glutathione 5-Transferase (GST)-MDA-S Fusion Protein. A baculovirus transfer vector of GST-MDA-5 was constructed by ligation of the XhoI-BamHl *mda*-5 fragment from the pEGFP-C2/*mda*-5 and XhoI BgIII sites of pAcGHLT-C (PharMingen). The transfer vectors of GST only and GST-MDA-5 fusion (5 μg each) were transfected into Sf9 cells, and recombinant virus producer cells were selected as described by the manufacturer (PharMingen). GST and GST-MDA-5 fusion proteins were expressed and purified by affinity chromatography with glutathione-Sepharose 413 as described by the manufacturer (PharMingen).

[0079] ATPase Assay. ATPase assays were performed in a 10-μ1 reaction mixture containing 50 mM Mops, pH 6.5, 3 mM $MgCl_2$, 2 mM DTT, 0.5 mM ATP, 5 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP (6,000 Ci/mmol; Amersham Pharmacia), DNA (sonicated salmon sperm) or RNA, and protein (0.2 μg) in a 37°C air incubator. Poly(I)•poly(C) (Amersham Pharmacia) and other RNA samples were reextracted with acid phenol /chloroform and precipitated. Specific assay conditions are described in the figure legends. The reaction was started by addition of the ATP mixture and the dissociated P_i was resolved in polyethylene imine cellulose TLC plates as described (Wagner *et al.*, 1998; *EMBO J* 17:2926-2937).

6.2 RESULTS AND DISCUSSION

[0080] IFN-ß + MEZ Induces Terminal Differentiation in HO-1 Cells. Treatment of HO-1 melanoma cells with IFN-ß (2,000 units/ml) plus MEZ (10 ng/ml) results in rapid and irreversible growth suppression, induction of dendrite-like processes, and enhanced melanogenesis (Fisher PB *et al.*, 1985, *J Interferon Res* 5:11-22; Jiang H *et al.*, 1993, *Mol Cell Different* 1:41-66). This combination treatment also results in a subset of detached cells that display A_o DNA content by fluorescence-activated cell sorter analysis, which is indicative of apoptosis. These floating cells are apparent first at 48 h. In contrast, treatment with either agent alone inhibits growth and melanogenesis in a reversible manner, and treated cells do not terminally differentiate or undergo apoptosis. In this context, induction of terminal differentiation in HO-1 cells by the combination treatment would be anticipated to involve the induction of genes that are relevant to the mode of action of the individual inducing agents, *i.e.* IFN-ß and MEZ, related to both reversible and terminal differentiation, and associated with apoptosis (Jiang and Fisher, 1993, *Mol Cell Different* 1:285-299; Jiang H *et al.*, 1993, *Mol Cell Different* 1:41-66; Leszczyniecka *et al.*, 2001, *Pharmacol Ther* 90(2-3):105-156).

[0081] Sequence Analysis of *mda*-5. The cloned *mda*-5 cDNA [3,365 bp, excluding the poly(A) tail] contains a 1,025-amino acid-encoding ORF (nucleotides 169-3,246) with a predicted molecular mass of 116.7 kDa and a pI of 5.44. The first AUG at position 169 likely represents a genuine start codon caused by the presence of an in-frame stop codon at nucleotide 159 and an A^{-3} Kozak consensus sequence (AXXaugG; Kozak M, 1996, *Mamm Genome* 7:563-574). A polyadenylation signal (AATAAA) is located 23 by upstream of the poly(A) tail. *In vitro* translation of the

mda-5 cDNA resulted in a protein with apparent molecular mass of 120 kDa and additional smaller bands, most likely truncated translation products of MDA-5.

[0082] Sequence analysis of the MDA-5 protein in the Prosite database identities two conserved domains, a DExH/D RNA helicase domain and a CARD. No nuclear localization signal or RNA binding domains were found in this protein. The C-terminal 102 amino acids (nucleotides 722-823) of MDA-5 show homology to the RNA helicase C-terminal conserved domain, a signature motif of helicase superfamily II, which consists of three subfamilies (DEAD, DEAH, and DExH) based on their ATPase B motif (Lüking *et al.*, 1998, *Crit Rev Biochem Mol Biol* 33(4):259-296; Jankowsky and Jankowsky, 2000, *Nucleic Acids Res* 28:333-334). At least eight of nine motifs are well conserved among DExH group helicases (Jankowsky and Jankowsky, 2000, *Nucleic Acids Res* 28:333-334). These eight DExH group helicase motifs display high conservation but show some divergence in MDA-5 (numbered in FIGURE 4). The distance between consensus motifs, which also are well conserved in the DEAD group helicases (Lüking *et al.*, 1998, *Crit Rev Biochem Mol Biol* 33(4):259-296), are extended further in MDA-5 (FIGURE 4). In these contexts, MDA-5 seems to be a divergent form of DExH RNA helicase.

[0083] The ProDom database recognizes 10 cloned or hypothetical proteins closely related in their helicase domain to MDA-5 (dating back to early prokaryotes: *Methanobacterium thermoautotrophicum*, 027466; *Schizosaccharomyces pombe*, Q09884; *Caenorhabditis elegans*, Q17545, Q44165, and P34529; *Arabidopsis thaliana*, Q9SP32; *Sus scrofa*, RHIV-1; and *Homo sapiens*, RIG-1, Q9HAM6, and Q9UPY3). Proteins except RIG-7 (Sun YW, 1995, In *Sanghai Institute of Hematology. Rui-Jin Hospital* (Sanghai Second Medical University, Sanghai, China)),

RHIV-1 (Zhang *et al.*, 2000, *Microb Pathog* 28:267-278), and Q9SP32 (Jacobsen *et al.*, 1999, *Development* 126:5231-5243) contain hypothetical ORFs deduced from genomic sequence analysis of diverse species. CLUSTALW sequence alignment of representatives of these proteins (MDA-5, RIG-1, Q9HAM6, RHIV-1, P34529, Q9SP32, and Q09884) revealed that the DExH helicase motifs found in MDA-5 and 10 additional motifs/residues are well conserved within this group of proteins (FIGURE 4). The greatest sequence homology to the MDA-5 helicase domain was observed with Q9HAM6, a hypothetical ORF from the human genome. The conserved pattern of helicase motifs among these molecules possibly signifies a newly identified subgroup of DExH RNA helicases. Among them, MDA-5 is the first protein with documented RNA-dependent ATPase activity (FIGURE 8).

[0084] RNA helicases impinge on many biological phenomena including cell differentiation, proliferation, development, and viral life cycle (Lüking *et al.*, 1998, *Crit Rev Biochem Mol Biol* 33(4):259-296; Jankowsky and Jankowsky, 2000, *Nucleic Acids Res* 28:333-334). Although RNA helicases are well conserved in their ATPase and helicase motifs, a direct correlation between sequence motifs and function is not clear-cut. In this context, it is not possible to deduce the potential functions) of MDA-5 based on its primary structure. However, it is worth noting that four of the molecules (Q93P32, Q09884, P34529, and Q9UPY3) of this group contain an RNase III motif as well as an RNA helicase domain. RNase III is an enzyme targeting double-stranded RNA and is involved in gene silencing by RNA interference (Bernstein *et al.*, 2001, *Nature* 409(6818):363-6). Thus, it is conceivable that MDA-5, through its ATP-dependent unwinding of RNA, most likely functions to promote message degradation by specific types of RNase.

[0085] The biological context of expression and function of the four cloned proteins (MDA-5, RIG-1, RHIV-1, and Q9SP32) are strikingly similar. RIG-1 is expressed during *all-trans* retinoic acid-induced promyelocytic differentiation (Sun YW, 1995, In *Sanghai Institute of Hematology. Rui-Jin Hospital* (Sanghai Second Medical University, Sanghai, China)) and also is induced by IFN-ß in HO-1 cells. The expression of RHIV-1 is induced by viral infection suggesting IFN inducibility (Zhang *et al.*, 2000, *Microb Pathog* 28:267-278). In addition, Q93P32 (also known as CAF) seems to suppress cell division in floral meristems (Jacobsen *et al.*, 1999, *Development* 126:5231-5243). *mda*-5 is expressed during differentiation induced by IFN-/3 plus MEZ, which also involves growth inhibition. In this regard, it is possible that the putative helicases of this subgroup may participate in similar biochemical changes associated with growth inhibition and differentiation.

[0086] A distinctive attribute of MDA-5 involves the N-terminal one-third of this molecule. This region of the MDA-5 protein contains a CARD (amino acids 125-174), with significant but relatively low probability (P = 0.04). The CARD is a structural motif that consists of amphipathic α-helices and is present in various pro- and antiapoptotic molecules (Hofmann *et al.*, 1997, *Trends Biochem Sci* 22:155-156). The recruitment of caspase to apoptotic signaling receptor complexes through CARD-CARD interactions has been documented (Chou *et al.*, 1998, *Cell* 94:171-180). Although the N-terminal 50 amino acids (125-174) of MDA-5 do not align with other CARD proteins, probably because CARD is a structural motif, secondary structure analysis of amino acids 101-200 of MDA-5 with secondary structural content prediction indicates 83.3% α-helical contents in this area. Based on these

considerations, it is likely that the MDA-5 N terminus contains a CARD, and would represent the first RNA-modulating molecule associated with programmed cell death.

[0087] *mda*-5 Expression Analysis. Because *mda*-5 was cloned in the context of induction of growth arrest and both reversible and terminal differentiation in HO-1 cells, experiments were performed to determine the effect on expression of agents affecting these parameters. Of the agents tested that influence melanocytic differentiation in human melanoma cells (Kang *et al.*, 2001, *Gene* 267:233-242; FIGURE 5A) and modulate melanoma growth (Garbe *et al.*, 1993, *J Invest Dermatol* 100:239S-244S; FIGURE 5B), only IFNs (α, ß, and γ) and TNF-α significantly increased steady-state *mda*-5 transcript levels within 24 h. However, the magnitude of induction by IFN-ß was at least 3-fold greater than with IFN-γ, IFN-α, and TNF-α. Induction of DNA damage by exposure to the alkylating agent methyl methanesulfonate or growth in serum-free medium for 24 h did not induce *mda*-5 expression. Although MEZ treatment for 24 h by itself did not induce *mda*-5 expression, it augmented *mda*-5 expression by 1.5-fold when used in combination with IFN-ß or IFN-γ (FIGURE 5A). Although *mda*-5 is an IFN- and TNF-α-responsive gene, it responds primarily to IFN-ß treatment. Because the other differentiation/growth-regulating reagents tested were not effective inducers of *mda*-5 expression, the primary role of *mda*-5 in the induction of terminal cell differentiation of HO-1 cells by IFN-ß + MEZ seems to be restricted to IFN-β-mediated suppression of cell proliferation and/or induction of apoptosis (Stark *et al.*, 1998, *Annu Rev Biochem* 67:227-264).

[0088] The timing of *mda*-5 expression was studied also by Northern blotting, indicating induction of *mda*-5 mRNA by 2 h of treatment with IFN-ß or IFN-ß +

MEZ. The *mda*-5 message level peaked between 6 and 8 h and remained elevated over a 96-h period. Although MEZ further increased *mda*-5 message level above that observed with IFN-ß alone, it did not affect the timing of *mda*-5 expression. Pretreatment with the protein synthesis inhibitor cycloheximide (CHX) did not inhibit the induction of *mda*-5 by IFN-ß or IFN-ß + MEZ. These results, which correspond with the early onset of *mda*-5 expression induced by these reagents, provide support for *mda*-5 being an early response gene induced without prior protein synthesis. They also indicate that *mda*-5 plays a critical role during an early stage of IFN-induced biological response. The organ-specific expression pattern of *mda*-5 was determined by hybridization with multiple tissue Northern blots (CLONTECH). Most organs expressed *mda*-5 at low levels except the brain, testis, and lung, in which expression was barely detectable. Although *mda*-5 expression was ≈2-fold higher in placenta, pancreas, and spleen than other organs, no organ manifested significantly high enough expression to suggest a correlation between *mda*-5 expression and specific organ function. Northern blot analysis of *mda*-5 expression in cancer and normal cell lines of various sources again indicated low basal levels of expression with strong induction by IFN-ß.

[0089] Treatment of human skin fibroblasts with IFNs or TNF-α induces and MEZ augments *mda-5* expression in a similar manner as in HO-1 cells. These results suggest that induction of *mda*-5 by these agents may represent a general response of this gene occurring in different cellular contexts. Among the cytokines inducing *mda*-5 expression, IFN-5 is the most potent. Gene expression profiles induced by type I IFNs (α/ß), which bind to common receptors and share transcription factors, are quite similar, but the magnitude of differential gene expression and growth-inhibitory

effects induced by IFN-ß are more profound, as observed with *mda*-5 induction (Kang *et al.*, 2001, *Gene* 267:233-242; Stark *et al.*, 1998, *Annu Rev Biochem* 67:227-264; Garbe *et al.*, 1990, *J Invest Dermatol* 95:231S-237S). Although differences have been reported in tyk2 requirement and specific coprecipitation of IFNARI with IFN-ß but not with IFN-α, the mechanism underlying these differences in signal transduction between type I IFNs is not well understood (Stark *et al.*, 1998, *Annu Rev Biochem* 67:227-264). IFN-γ and IFN-γ + MEZ induce 3-fold lower levels of *mda*-5 mRNA than IFN-ß and IFN-ß + MEZ, but these treatments also directly induce *mda*-5 expression without prior protein synthesis. It is possible that the *mda*-5 promoter contains an IFN-γ response sequence such as GAS (Stark *et al.*, 1998, *Annu Rev Biochem* 67:227-264). Alternatively, the signal generated by IFN-γ may impact on *mda*-5 expression through shared signaling components with IFN-ß but not through IFN-ß production. In 3T3-Ll adipocytes, TNF-α activates the Janus kinase/signal transducer/activator of transcription signal transduction (Jak/STAT) pathway, which is stimulated by type I IFNs (Guo *et al.*, 1998, *J Immunol* 160:2742-2750). TNF-α may induce *mda*-5 expression in HO-1 cells by activation of Jak/STAT signals as found in 3T3-Ll cells. Therefore, the Jak/STAT signal transduction pathway seems to be a major contributor to *mda*-5 expression.

[0090] IFNs were identified initially as molecules that provide immediate protection against viral infection by eliciting an antiviral state in exposed cells (Stark *et al.*, 1998, *Annu Rev Biochem* 67:227-264). IFN treatment evokes diverse responses depending on the target cell including growth inhibition, changes in differentiation, induction or inhibition of apoptosis, and changes in the expression of immune system modulating genes (Leszczyniecka *et al.*, 2001, *Pharmacol Ther* 90(2-3):105-56; Stark

et al., 1998, *Annu Rev Biochem* 67:227-264; Grant *et al.*, 1985, *Biochem Biophys Res Commun* 130:379-388; Greiner *et al.*, 1985, *Pharmacol Ther* 31:209-236). The highly inducible nature of *mda*-5 expression by IFNs regardless of cell type, especially IFN-ß, the relatively low basal message level in various organs, and the rapid response to IFN treatment strongly suggest that *mda*-5 plays a critical role in responses that are specific for IFN signaling such as antiviral effect, growth inhibition, and apoptosis but may be less critical during normal physiological processes.

[0091] Although CARD interactions were not reported previously in IFN signaling, IFNs and their biological effectors do sensitize or induce apoptosis in specific cellular contexts, suggesting a link between IFN and apoptotic signaling (Stark *et al.*, 1998, *Annu Rev Biochem* 67:227-264; Balachandran *et al.*, 2000, *J Virol* 74:1513-1523). Coincidentally, although not as effective as IFN-ß, TNF-α, an apoptotic cytokine, also induces *mda*-5 expression in both HO-1 and human fibroblasts (Wallach *et al.*, 1999, *Annu Rev Immunol* 17:331-367; FIGURE 5B). Hence, MDA-5 may interact with death molecules through CARD and participate in the IFN- and/or TNF-α-induced apoptotic process by modulating RNA structure. MDA-5 also could have multiple functions with one or more of its potential enzymatic activities predominating, depending on the cellular context and presence of other coeffectors, *e.g.* CARD-containing proteins.

[0092] Nuclear run-on assays of HO-1 cells treated with IFN-ß confirmed increases in *mda*-5 transcription in comparison with undetectable levels of transcription in untreated or MEZ-treated cells (FIGURE 5C). The combination of IFN-ß + MEZ further enhanced the transcription level of *mda*-5 as compared with IFN-ß alone, an observation compatible with effects on steady-state message levels. These findings

suggest that MEZ, in the combinatorial treatment protocol, augments *mda*-5 expression induced by IFN-ß at a transcription level. Analysis of *mda*-5 mRNA stability by using actinomycin D to block transcription revealed that the half-life of the *mda*-5 transcript was ≈5 h and the decay rate of *mda*-5 message was indistinguishable between single- and combination-treated cells. These data document that the increased steady-state levels of *mda*-5 message by IFN-ß and IFN-ß + MEZ treatment are primarily the result of increased *mda*-5 transcription.

[0093] The ability of IFN-ß + MEZ to potentiate *mda*-5 mRNA levels suggests cooperativity between IFN and MEZ signaling in regulating *mda*-5 expression. Because MEZ is an activator of PKC, a potential role for PKC in enhancing *mda*-5 expression after IFN-ß + MEZ treatment was tested by pretreatment of HO-1 cells with PKC-specific inhibitors, staurosporine or Ro31-8220 (Tamaoki *et al.*, 1986, *Biochem Biophys Res Commun* 135:397-402; Morreale *et al.*, 1997, *FEBS Lett* 417:38-42; FIGURE 5D). Both reagents abolish the enhanced *mda*-5 induction by combination treatment. These findings indicate that both IFN and PKC signaling pathways are required for maximal induction of *mda*-5 expression in HO-1 cells. However, because MEZ does not always augment gene expression induced by IFN-ß (*e.g.* human UBP43; Kang *et al.*, 2001, *Gene* 267:233-242), crosstalk between PKC and IFN signaling pathways for all up-regulated genes seems unlikely. The higher level of induction seen in combination-treated cells seems to be through direct but independent action on the *mda*-5 promoter.

[0094] Intracellular Localization of MDA-5. A GFP-MDA-5 fusion protein was transiently expressed in 293 cells and Western blot analysis of cell lysates detected a predicted ≈160-kDa protein (GFP-tagged) in *mda*-5 cDNA-transfected cells (FIGURE

6A). Confocal fluorescence microscopy of 293 cells transiently transfected with GFP-MDA-5 fusion protein demonstrated that the protein localizes in the cytoplasm (FIGURE 6B). The cytoplasmic localization of the MDA-5 protein corresponded with the absence of a nuclear localization signal in the MDA-5 protein. No specific localization pattern within the cytoplasm of the GFP-MDA-5 fusion protein was apparent. Cytoplasmic localization of MDA-5 suggests that MDA-5 plays a role in the translation, sequestration of specific mRNAs, or in regulating mRNA stability.

[0095] *mda*-5 Inhibits the Colony-Forming Ability of HO-1 Cells. Treatment of HO-1 cells with 2,000 units/ml of IFN-ß for 4 days results in ≈60% growth inhibition versus untreated controls (Fisher *et al.*, 1985, *J Interferon Res* 5:11-22; Jiang *et al.*, 1993, *Mol Cell Different* 1:41-66). Because *mda*-5 is induced primarily by IFN-ß in HO-1 cells, ectopic expression of *mda*-5 could mimic the effect of IFN-ß treatment and inhibit growth. To test this possibility, the *mda*-5 gene was expressed in HO-1 cells by transfection, and colony-forming ability was determined (FIGURE 7A and B). Expression of the *mda*-5 construct was confirmed in 293 cells, which are more efficient for transfection than HO-1 cells. Western blot analysis using α-HA antibody showed a protein band corresponding to the predicted size of MDA-5 (≈120 kDa) in HA-*mda*-5-transfected cell lysates (FIGURE 6A). After verifying HA-MDA-5 expression, colony-forming efficiency of HO-1 cells was determined after transfection with parental vector, HA-*mda*-5 expression vector, or an antisense *mda*-5 expression vector. The number of G418-resistant HO-1 colonies developing after transfection with the *mda*-5 expression vector was reduced significantly (≈67%, $P < 0.05$) in comparison with cells transfected with parental vector (FIGURE 7B). In contrast, transfection with the antisense *mda*-5 expression vector did not effect colony

formation significantly, which supports a specific role of *mda*-5 in regulating the growth of HO-1 cells. Moreover, attempts to obtain stable HO-1 cell clones expressing *mda*-5 have proven unsuccessful thus far, providing substantiation for a growth-inhibitory effect of *mda*-5 in these cells.

[0096] ATPase Activity of MDA-5. A GST-fusion protein of MDA-5 was purified by glutathione-Sepharose affinity chromatography and the purified GST-MDA-5 migrates as a single protein in SDS-PAGE with an apparent molecular mass of 170 kDa (FIGURE 8A). GST as a control was expressed and purified in a manner similar to that for GST-MDA-5 (FIGURE 8A). The ATPase activity of the MDA-5 fusion protein was 2-fold more active at pH 6.5 than at pH 7.5 but strictly depended on poly(I)•poly(C) (primarily exists as a double-stranded molecule) at both pH values. Poly(I)•poly(C) has been shown to induce *mda*-5 in HO-1 and other cancer cells. The requirement of divalent metal ions and effective RNA concentrations for MDA-5 ATPase activity were determined at various poly(I)•poly(C) concentrations by using either Mg^{2+} or Mn^{2+} (FIGURE 8B). The presence of Mn^{2+} shifted the optimal poly(I)•poly(C) concentration for MDA-5 ATPase activity to a higher range. ATPase activity peaked at 3.75 ng of poly(I)•poly(C)/μl with Mg^{2+} and at 37.5 ng of poly(I)•poly(C)/μl with Mn^{2+}. The specific activity of MDA-5 at optimal conditions was calculated as 1.01 ± 0.22 nmol•min•μg of protein with Mg^{2+} and 0.80 ± 0.03 nmol•min•μg of protein with Mn^{2+}. These results suggest that Mg^{2+} is likely to be the preferred ion for MDA-5. Unexpectedly, increasing poly(I)•poly(C) concentrations did not increase MDA-5 ATPase activity. At the highest poly(I)•poly(C) concentration tested, ATPase activity of MDA-5 was reduced to 0.20, ≈0.25-fold of peak activity. Depletion of divalent metal ions by excessive poly(I)•poly(C) provides

a possible explanation for this phenomenon. ATPase activity of MDA-5 also was determined at various ATP concentrations, and the calculated K_m value for ATP as determined by double reciprocal plot between [ATP] and hydrolyzed P_i fraction was 26 μM (FIGURE 8C). The K_m value of MDA-5 for ATP is lower than other well characterized RNA helicases including eIF4A (DEAD helicase, 160 μM; Pause *et al.*, 1993, *Mol Cell Biol* 13:6789-6798), NPH-11 (DEAH helicase, 1.2 mM) (Gross and Shuman, 1996, *J Virol* 70:1706-1713.), and hepatitis C virus NS3 protein (DECH helicase, 42 μM without RNA; Kim *et al.*, 1997, *J Virol* 71:9400-9409.). Based on these results, MDA-5 is strictly an RNA-dependent ATPase, the activity of which may be limited more by the presence of double-stranded RNA than by [ATP]. These results also reinforce the suggestion that MDA-5 is an ATP-dependent RNA helicase.

[0097] To identify a potential target of MDA-5, the ATPase activity of MDA-5 was measured in the presence of cellular RNA samples. Of interest, no significant ATPase activity was observed with total, either poly(A) or poly(A)-negative RNA, from IFN-treated HO-1 cells and yeast tRNA (20 ng/μl in FIGURE 8D; 375 ng/μl). The lack of a stimulatory effect on MDA-5 ATPase activity by cellular RNA may reflect the heterogeneous nature of the cellular RNA populations or the extent of double-stranded RNA. However, although rRNA and tRNA are highly double-stranded and relatively homogeneous in comparison with poly(A) RNA, they did not activate MDA-5 ATPase activity, thereby ruling out these molecules as potential substrates for MDA-5. ATPase activity was apparent also with poly(A, U), which was significantly higher than with poly(I, U), suggesting no specific preference for inosinic acid.

[0098] Various publications, including journal articles, electronic documents, patents and patent applications are cited herein, the contents of which are hereby incorporated by reference in their entireties.